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Physiology

Morphine-like peptides in mammalian brain: Isolation, structure elucidation, and interactions with the opiate receptor

(methionine/leucine/naloxone)

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ABSTRACT **A substance that competes for opiate receptor binding has been isolated from calf brain and identified as two pentapeptides, H-Tyr-Gly-Gly-Phe-Met-OH (methionine enkephalin) and H-Tyr-Gly-Gly-Phe-Leu-OH (leucine enkephalin), with about four times more leucine enkephalin than methionine enkephalin. Sodium and manganese effects on opiate receptor interactions show that both peptides are agonists, whereas leucine enkephalin may be a "purer" agonist than methionine enkephalin.**

Specific opiate receptor binding (1–3) portended the existence of a "natural" opiate "enkephalin" that was demonstrated in brain by its effects on smooth muscle (4, 5) and by its competition for receptor binding (6–9), whereas pituitary extracts contain a different opiate-like substance (10). Regional (4, 8, 9, 11, 12) and subcellular (13) localizations of enkephalin parallel opiate receptor binding. Hughes *et al.* (14) identified pig brain enkephalin as two pentapeptides, Tyr-Gly-Gly-Phe-Met (methionine enkephalin, Met-enkephalin) and Tyr-Gly-Gly-Phe-Leu (leucine enkephalin, Leu-enkephalin), with about four times more Met-enkephalin than Leu-enkephalin. In bovine brain we independently isolated and identified the same two peptides with four times more Leu-enkephalin than Met-enkephalin (15), here described in detail.

MATERIALS AND METHODS

Extraction and Purification of Enkephalin. Fresh calf brains minus cerebella, homogenized by Polytron (Brinkmann) in 2–5 volumes of 10 mM Tris·HCl buffer (pH 7.7 at 25°), were centrifuged at 100,000 × *g* for 60 min, and the supernatant was heated for 15 min in a boiling-water bath. The boiled homogenate was centrifuged at 100,000 × *g* for 60 min and the supernatant was lyophilized. The powder was extracted in methanol (20 vol/wt) and the methanol was evaporated at 30°. The residue, dissolved in 20 ml of 50 mM Tris·HCl buffer (pH 7.7 at 25°), was applied to a Biogel P_2 column (95 × 3.1 cm) that was prepared and eluted in the same buffer. Fractions 21–29 were pooled, lyophilized, and resuspended in 20 ml of 100 mM NH_4-acetate buffer, pH 7.4, applied to a column of AG-1-X2 (200–400 mesh, Bio-Rad, 35 × 1.8 cm), and prepared and washed with 150 ml of the NH_4-acetate buffer. The column was eluted with a linear pH gradient of 250 ml of 100 mM NH_4-acetate buffer, pH 7.4, and 250 ml of 100 mM acetic acid. Fractions (7 ml) with peak opioid activity (eluted at pH 5.6–5.9) were pooled, lyophilized, suspended in 10 mM HCl, and applied to a column of AG-50W-X2 (200–400 mesh, Bio-Rad, 25 × 1.1 cm) prepared in 10 mM HCl. Fractions were eluted, first successively with 100 ml of 10 mM HCl and 100 ml of 100 mM NH_4-acetate buffer, pH 7.4, and then with a linear pH gradient of 250 ml of 100 mM NH_4-acetate buffer, pH 7.4, and 250 ml of 100 mM NH_4OH. Five-milliliter fractions were lyophilized, suspended in 0.5 ml of H_2O, and assayed for opiate receptor activity. The peak fractions were applied to an Amberlite CG-400 column (26 × 1.1 cm), prepared in 200 mM NH_4-formate buffer, pH 8.5, washed with 100 ml of 200 mM NH_4-formate buffer, pH 8.5, and then eluted with a linear pH gradient of 200 ml of 0.2 M NH_4-formate buffer (pH 8.5) and 200 ml of 0.2 M formic acid. After lyophilization, peak fractions (10–50 μl) were applied to 3 MM Whatman paper and subjected to high-voltage paper electrophoresis at 30 V/cm for 90 min at pH 2 (8% acetic acid, 2% formic acid). Material was extracted from strips in 2.5 ml of 10% acetic acid. The extracts were lyophilized, suspended in 0.5 ml of H_2O, and assayed for enkephalin activity. Eluates of the Biogel P2, the AG-1-X2, and the AG-50W-X2 columns were monitored for "protein" by UV absorbance at 280 nm. Samples from Amberlite CG-400 and the paper electrophoresis steps were assayed by the fluorescamine method (16) with glycine as a standard.

Determination of Amino Acid Composition and Sequence. Two nanomoles of enkephalin, purified through the electrophoresis step (fluorescamine, glycine equivalents), were hydrolyzed with 5.5 M constant boiling HCl (Pierce Co.) for 2–18 hr at 105°. Amino acid composition of the hydrolysate was determined after dansylation by chromatography on Cheng-Chin polyamine layer sheets (17) with 18 standard dansylated amino acids (Pierce Co.). For determination of tryptophan, samples were hydrolyzed with 3 M *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethylindole) (18) or with 3 M mercaptoethanesulfonic acid (19) at 110° for 48 and 72 hr.

Table 1. Purification of calf brain enkephalin

Step of purification	Enkephalin activity (unit/nmol of glycine equivalent)	Purification (–fold)
1. Brain extract	0.006	—
2. Methanol extraction	0.028	4.6
3. Biogel-P_2 gel chromatography	0.21	34.6
4. AG-1-X2 ion exchange	2.6	435
5. AG-50W0X2 ion exchange	30	5,019
6. Amberlite-CG-400 ion exchange	155	25,900
7. High-voltage paper electrophoresis, pH 2.0	292	48,600

Enkephalin activity was assayed as in *Materials and Methods*. The purification scheme was repeated three times, with less than 20% variation in extent of purification in all steps.

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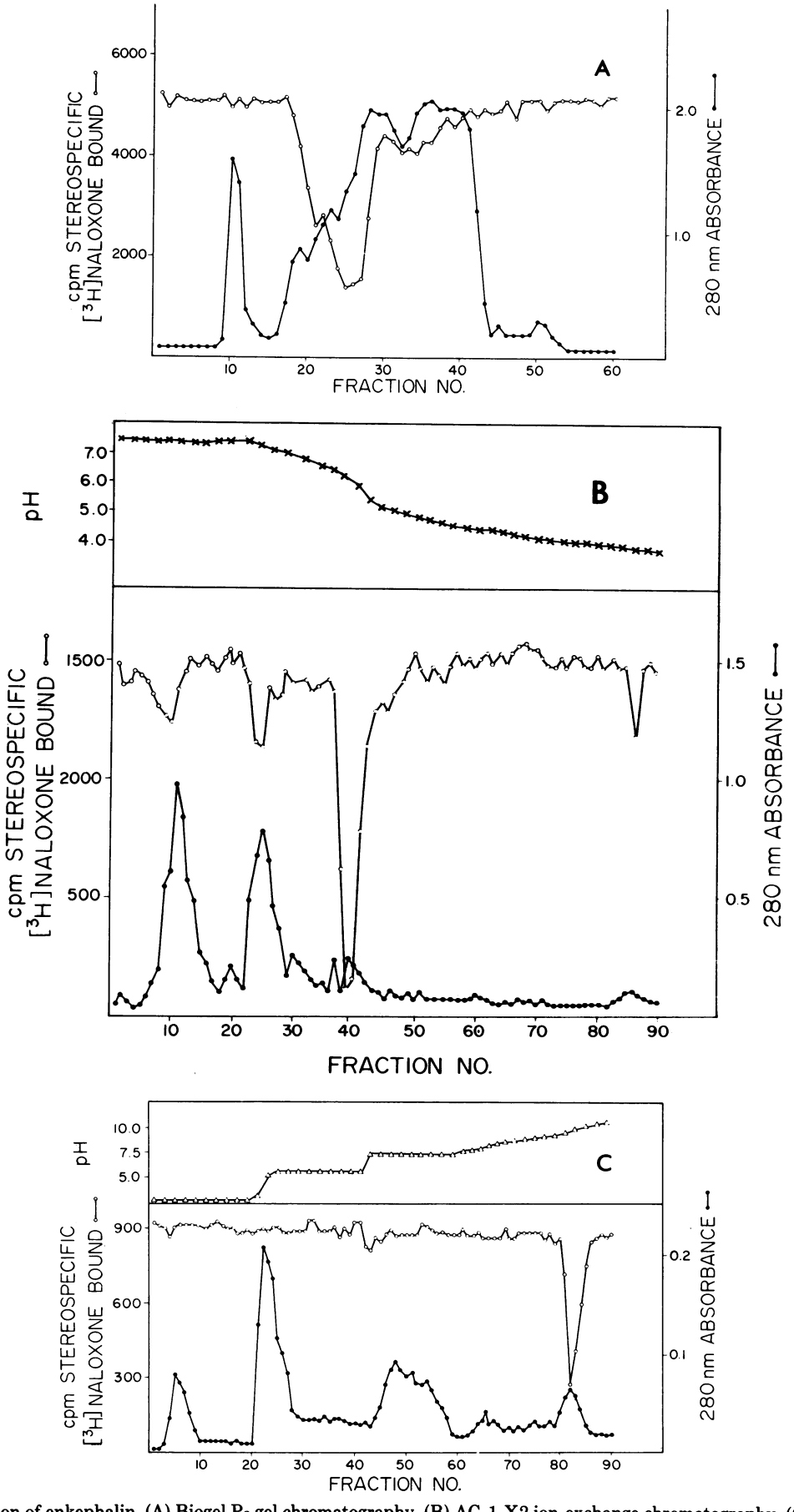

FIG. 1. Purification of enkephalin. (A) Biogel P_2 gel chromatography. (B) AG-1-X2 ion-exchange chromatography. (C) AG-50W-X2 ion-exchange chromatography.

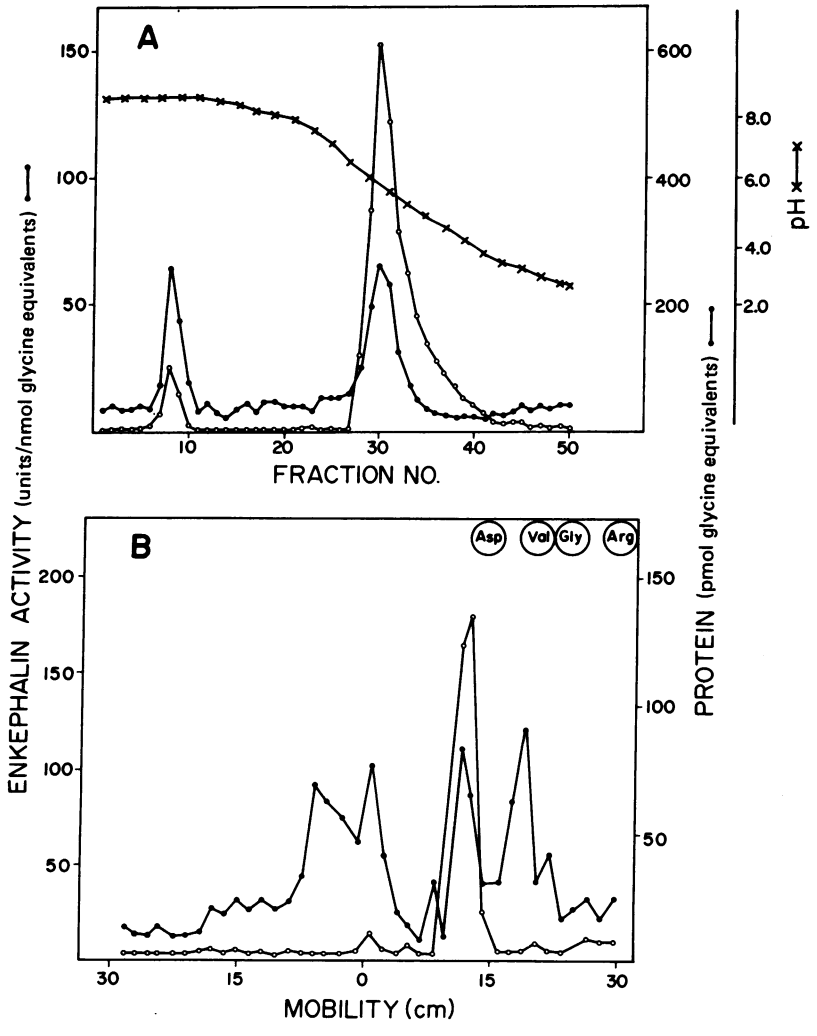

FIG. 2. Purification of enkephalin. (A) Amberlite CG-400 ion-exchange chromatography. (B) High-voltage paper electrophoresis.

The amino acid sequence was investigated by the sequential degradation dansyl-Edman procedure (20). The carboxyl-terminal was determined by incubating 10 nmol of enkephalin for 0–15 min in H_2O at 25° on a 0.5 × 3 cm column of carboxypeptidase A bound to Sepharose (Worthington Biochemical Co.). The eluate (10 ml) from the carboxypeptidase column was lyophilized and dansylated, and amino acids were determined by chromatography on polyamine sheets. Relative amounts of the dansylated amino acids were quantified by comparison to 0.01–1 nmol of standard dansylated amino acids. The polyamine sheets (7.5 × 7.5 cm) were placed in two solvents: (*i*) H_2O–90% formic acid (200:3, vol/vol), and (*ii*) benzene-glacial acetic acid (0:1, vol/vol). A third solvent, *n*-heptane–butanol–glacial acetic acid (3:3:1, vol/vol/vol) gave similar results.

Synthetic peptides Tyr-Gly-Gly-Phe-Leu and Tyr-Gly-Gly-Phe-Met were a generous gift of Dr. D. Hauser and Dr. F.

Table 2. Inhibition of [^{3}H]naloxone binding by enkephalin: Ionic effects

Ions	IC_{50} (nM)*				
	Natural enkephalin	Tyr-Gly-Gly Phe-Leu	Tyr-Gly-Gly-Phe-Met	Morphine	Naloxone
None	25	30	8	5	3
NaCl	150	200	30	30	3
KCl	27	32	9	5	3
$MnCl_2$	6	8	2.7	2	3
$CaCl_2$	23	30	7	2	3
Sodium shift†	6.0	6.7	3.75	6.0	1.0
Manganese shift	0.24	0.27	0.34	0.4	1.0

Ion concentrations were 100 mM for NaCl and KCl and 0.25 mM for $MnCl_2$ and $CaCl_2$.

* IC_{50} is concentration that inhibits [^{3}H]naloxone binding 50%.

† Sodium and manganese shifts, respectively, are the ratio of IC_{50} with NaCl to IC_{50} in its absence or the ratio of IC_{50} with manganese to IC_{50} in the absence of $MnCl_2$.

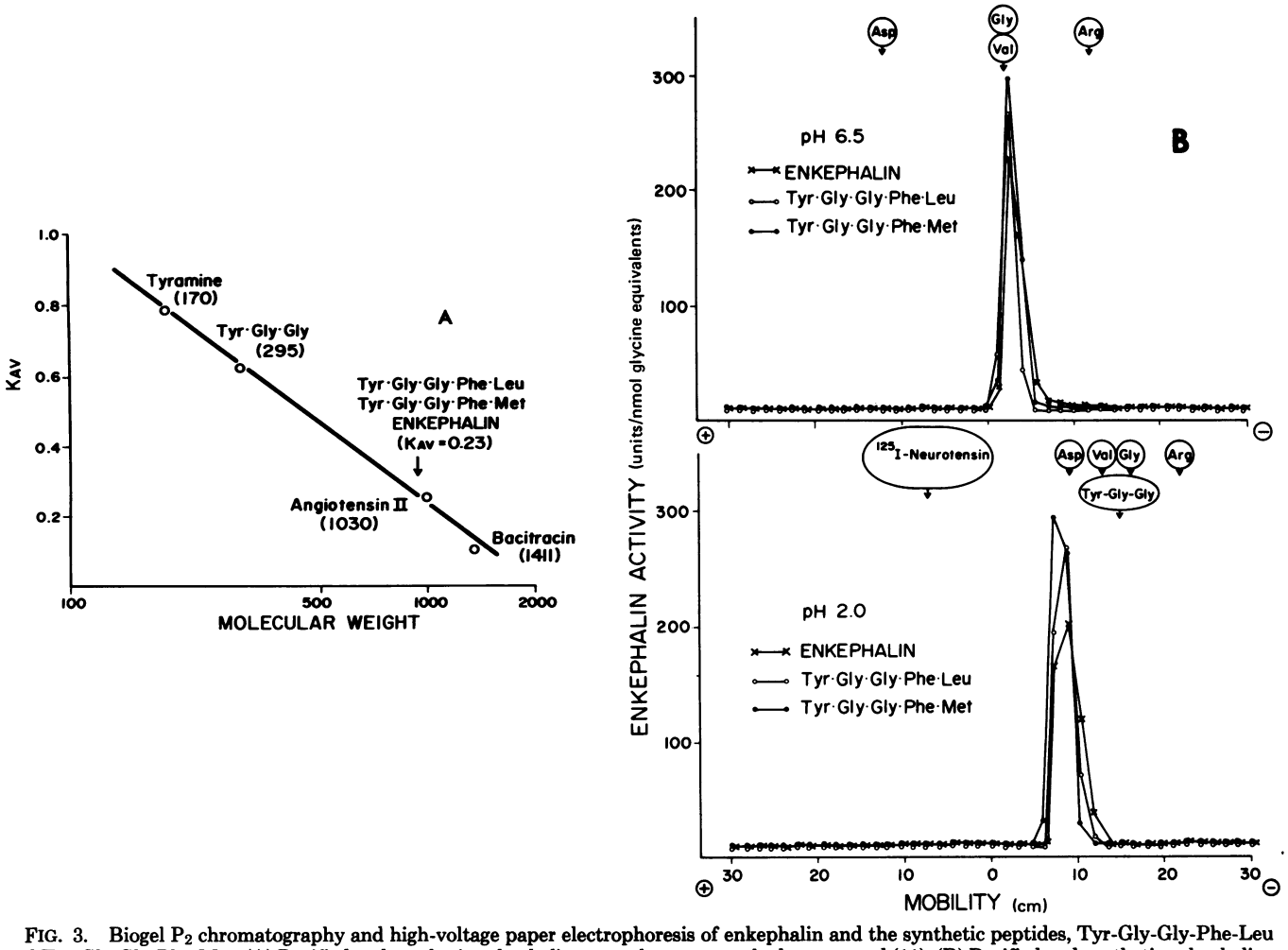

FIG. 3. Biogel P_2 chromatography and high-voltage paper electrophoresis of enkephalin and the synthetic peptides, Tyr-Gly-Gly-Phe-Leu and Tyr-Gly-Gly-Phe-Met. (A) Purified and synthetic enkephalins were chromatographed as reported (11). (B) Purified and synthetic enkephalins were subjected to electrophoresis as in *Materials and Methods*.

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Opioid activity was assayed as the ability to inhibit specific [^{3}H]naloxone (New England Nuclear Corp., 20 Ci/mmol) binding to rat brain membranes (21) as previously described (15). One unit of opioid activity "enkephalin" was defined as that amount that yields 50% occupancy in a 200-μl assay, according to Colquhoun (22).

RESULTS

Purification and Isolation of Enkephalin. We isolated apparently homogeneous enkephalin by a six-step purification. Almost all activity is extracted into absolute methanol, with a 4.6-fold purification (Table 1). Biogel P_2 columns give a further 7-fold purification. The AG-1-X2, AG-50W-X2, and Amberlite-CG-400 ion exchange columns provide successive enrichments of 13-fold, 12-fold, and 5-fold (Figs. 1 and 2; Table 1). Activity elutes from the Amberlite-CG-400 column in a single peak at pH 5.6–5.9 (Fig. 2), and subsequent high-voltage paper electrophoresis at pH 2 resolves enkephalin from other fluorescamine-reactive material, yielding apparently homogeneous enkephalin.

Assessment of Enkephalin Purity and Determination of Amino Acid Composition and Sequence. In Biogel P_2 chromatography, enkephalin purified through the electrophoresis step elutes as a single peak corresponding to the only peak of fluorescamine-reactive material, reflecting an apparent molecular weight of about 1000 (Fig. 3). High-voltage paper electrophoresis at pH 2 and pH 6.5 reveals single peaks of fluorescamine-reactivity and enkephalin activity. At pH 2, enkephalin activity migrates toward the anode slightly behind aspartic acid, indicating an approximate molecular weight of 600–800 (23), like pig brain enkephalin (5, 14). At pH 6.5, bovine brain enkephalin activity migrates very slightly toward the anode, like glycine and valine, so at this pH enkephalin lacks a strong charge (Fig. 3).

After dansylation and HCl hydrolysis a single spot on polyamine sheets corresponds to *bis*-tyrosine, indicating a single NH_2-terminal amino acid, tyrosine. Hydrolysis after dansylation with 3 M *p*-toluenesulfonic acid containing 0.2% 3-(2-amino-ethylinode) reveals no tryptophan.

Amino acid composition, assessed by dansylation after HCl hydrolysis and polyamine sheet chromatography, shows glycine, tyrosine, phenylalanine, leucine, and methionine. Spots for tyrosine, phenylalanine, and leucine are of equal intensity, whereas the spot for methionine is substantially weaker and that for glycine is more intense. Total hydrolysis with 3 M mercaptoethanesulfonic acid reveals no tryptophan.

The amino acid sequence was determined by dansyl-Edman sequence determination. The NH_2-terminal amino acid is confirmed as tyrosine, and the second, third, and fourth amino acids correspond to glycine, glycine, and phenylalanine, respectively. In repeated experiments the fifth amino acid in the sequence appears to consist of both leucine and methionine,

with the leucine spot about four times more intense than the methionine spot. Further treatment reveals no additional amino acids, suggesting that enkephalin may consist of two pentapeptides whose respective carboxyl terminals are leucine and methionine. Carboxyl-terminal determination by incubation with carboxypeptidase A bound to Sepharose for 5 or 8 min reveals a mixture of leucine and methionine, with about four times more leucine than methionine. If leucine were the carboxyl terminal and methionine were the next amino acid, further treatment with carboxypeptidase A should reveal an increase in methionine. However, incubation for 15 min fails to alter the relative amounts of leucine and methionine but reveals, in addition, phenylalanine in concentrations corresponding to those of leucine. These observations support the suggestions derived from the dansyl-Edman technique that there are two peptides whose respective carboxyl terminals are leucine and methionine. Bovine brain enkephalin activity is therefore attributable to two pentapeptides whose respective amino acid sequences are Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu, identical to sequences of pig brain enkephalins (14). However, whereas pig brain contains three to four times more Met-enkephalin than Leu-enkephalin, bovine brain possesses about four times more Leu-enkephalin than Met-enkephalin.

In Biogel P_2 chromatography, natural enkephalin elutes at an identical position as the synthetic Leu-enkephalin and Met-enkephalin (Fig. 3). In high-voltage paper electrophoresis at pH values of 2.0 and 6.5, natural enkephalin migrates as a single sharp peak, identical to those of synthetic Leu-enkephalin and Met-enkephalin (Fig. 3).

Interactions of Natural and Synthetic Enkephalins with the Opiate Receptor. Hughes *et al.* (14) found Met-enkephalin about three times as potent as morphine in inhibiting [^{3}H]naloxone binding in sodium-free homogenates of guinea pig brain. In the absence of sodium, native enkephalin inhibits [^{3}H]naloxone binding with an IC_{50} (median inhibitory concentration) of 24 nM, the same as Leu-enkephalin, whereas Met-enkephalin is about three to four times more potent (Table 2). Even at 1 μM, the tripeptide Tyr-Gly-Gly fails to alter [^{3}H]naloxone binding.

Sodium but not potassium decreases receptor binding of opiate agonists while enhancing binding of antagonists (24), whereas manganese but not calcium enhances agonist but not antagonist binding (25). At 10 mM NaCl natural and synthetic enkephalins become less potent in competing for [^{3}H]naloxone binding, an agonist profile, whereas competition by nonradioactive naloxone is unaffected. Potassium fails to alter the potencies of natural and synthetic enkephalins and morphine. With 0.25 mM manganese, morphine and synthetic and natural enkephalins become more potent in inhibiting [^{3}H]naloxone binding, whereas calcium is ineffective. Sodium diminishes potencies of natural enkephalin and synthetic Leu-enkephalin six to seven times, whereas manganese enhances potency 4-fold. By contrast, Met-enkephalin is reduced in potency only 3.75-fold by sodium and is enhanced in potency 3.0-fold by manganese. The closely similar effect of these ions upon natural enkephalin and Leu-enkephalin is consistent with our observation that about 80% of natural enkephalin consists of Leu-enkephalin. The more marked influences of sodium on receptor interactions of leucine than of methionine enkephalin suggest that Leu-enkephalin is a "purer" agonist than Met-enkephalin.

DISCUSSION

Guillemin *et al.* (26) identified a hexadecapeptide "α-endorphine" in pig neurohypophysis-hypothalamus incorporating the Met-enkephalin sequence and with guinea pig ileum potency resembling Met-enkephalin. Alpha-endorphine corresponds to fragments 61–76 of the pituitary peptide β-lipotropin (27–29).

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